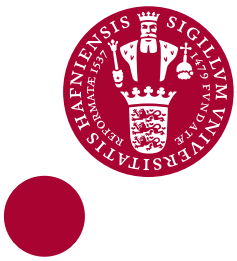

DNA repair as a human biomonitoring tool

Comet assay approaches

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DNA repair as a human biomonitoring tool; comet assay approaches

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Abstract

The comet assay offers the opportunity to measure both DNA damage and repair. Various comet assay based methods are available to measure DNA repair activity, but some requirements should be met for their effective use in human biomonitoring studies. These conditions include i) robustness of the assay, ii) sources of inter- and intra-individual variability must be known, iii) DNA repair kinetics should be assessed to optimize sampling timing; and iv) DNA repair in accessible surrogate tissues should reflect repair activity in target tissues prone to carcinogenic effects. DNA repair phenotyping can be performed on frozen and fresh samples, and is a more direct measurement than genomic or transcriptomic approaches. There are mixed reports concerning the regulation of DNA repair by environmental and dietary factors. In general, exposure to genotoxic agents did not change base excision repair (BER) activity, whereas some studies reported that dietary interventions affected BER activity. On the other hand, *in vitro* and *in vivo* studies indicated that nucleotide excision repair (NER) can be altered by exposure to genotoxic agents, but studies on other life style related factors, such as diet, are rare. Thus, crucial questions concerning the factors regulating DNA repair and inter-individual variation remain unanswered. Intra-individual variation over a period of days to weeks seems limited, which is favourable for DNA repair phenotyping in biomonitoring studies. Despite this reported low intra-individual variation, timing of sampling remains an issue that needs further investigation. A correlation was reported between the repair activity in easily accessible peripheral blood mononuclear cells (PBMCs) and internal organs for both NER and BER. However, no correlation was found between tumour tissue and blood cells. In conclusion, although comet assay based approaches to measure BER/NER phenotypes are feasible and promising; more work is needed to further optimize their application in human biomonitoring and intervention studies.

Keywords: DNA repair; comet assay; human biomonitoring; validation

1. Introduction

1.1. DNA damage and repair

Human DNA is exposed to both exogenous and endogenous agents that can modify its structure. These structural alterations can take different forms: breaks in the sugar-phosphate backbone affecting one or both strands [i.e., single strand breaks (SSBs) or double strand breaks (DSBs)], oxidation or alkylation of bases, large molecules covalently linked to DNA bases (bulky DNA-adducts), proteins linked to DNA bases (protein-DNA cross links), covalent bonds between bases in the same strand (intra-strand cross links) or in different strands (inter-strand cross links), and wrongly paired bases [1]. These DNA lesions can affect transcription but, more importantly, if not repaired or if mis-repaired before the replication process, they can induce mutations. Mutations in key genes (*e.g.* genes that control DNA repair, DNA replication, cell cycle control or chromosome segregation and apoptosis) are involved in the development of cancer and other degenerative diseases [2].

DNA repair systems, involving different groups of proteins, amend the majority of DNA damages before permanent genome changes can occur. Different DNA repair pathways deal with the various kinds of DNA lesions (see table 1). For instance, SSBs are repaired by the insertion of one or a few bases followed by ligation, while DSBs are repaired by more complicated processes, namely homologous recombination and non-homologous end-joining pathways (the latter being error-prone and therefore potentially mutagenic). Small base alterations such as oxidised and alkylated bases are predominantly repaired by the base excision repair system (BER), involving removal of the damaged base by a specific glycosylase, excision of the resulting baseless sugar, insertion of correct nucleotides using the opposite strand as template and ligation. More complex lesions such as bulky adducts, inter- and intra-strand cross links, and protein-DNA cross links are repaired by the nucleotide excision repair system (NER), in which an oligonucleotide containing the damage is excised and replaced with the correct nucleotides. Finally, wrongly paired bases are repaired by the mismatch repair system. For more details on each DNA repair mechanism, we refer to two comprehensive reviews [1, 4].

DNA repair activity is regarded as a valuable human biomarker, reflecting susceptibility to the accumulation of mutations and thus to cancer, the assumption being that a high intrinsic repair activity will reduce the likelihood of damage being present at replication. Repair activity is frequently assessed by measuring the level of transcription of selected genes from different DNA repair pathways or by the detection of gene polymorphisms (the latter often have

unknown consequences). However, the activity of an enzyme does not entirely depend on transcription and DNA repair is actually regulated in a post-translational manner, so a phenotypic or functional assay is more direct. Moreover, phenotypic analyses take into account the influence of environmental factors. Different phenotypic approaches based on the comet assay have been used to monitor DNA repair in human samples, but the question remains whether these assays are suitable for application in human biomonitoring studies.

The COST Action hCOMET ('The comet assay in human biomonitoring', CA15132, http://www.hcomet.org) with more than 60 researchers from 25 countries is addressing the application of the comet assay (single cell gel electrophoresis) to measure both DNA damage and DNA repair in human samples. This article has been prepared in the framework of this project as a starting point for further validation trials of the comet assay for assessing DNA repair activity. A working group on DNA repair measurements using the comet assay has identified the required conditions for using DNA repair phenotyping in human biomonitoring studies. These conditions include: 1) technical robustness of the assay; analysis of DNA repair activity by the comet assay must have advantages compared to other techniques; 2) sources of inter- and intra-individual variability must be identified; 3) DNA repair kinetics should be assessed to optimize sampling timing; and 4) DNA repair in accessible surrogate tissues should reflect repair activity in target tissues (i.e. tissues prone to carcinogenic effects). Here we describe the current status of these aspects in the scientific literature.

In this review, we predominantly included human biomonitoring studies that focussed on assessing DNA repair activity by comet assay approaches (see next section) in easily accessible tissues or cells. The comet assay-based repair assays are continuously and successfully being validated while at the same time being applied in various research studies. Scientific achievements emerge at the same time as initiatives to understand the assays, improving their reliability, and extending the applications to new tissues. The status of the assays is not advanced to a state where standardized protocols have been adopted. Substantial heterogeneity exists between studies, which very much depends on variation in assay conditions [5]. Although, meta-analysis is an integrate part of systematic reviews, the present variability in the comet assay-based DNA repair assays precludes a meaningful meta-analysis. Thus, only qualitative outcomes of the individual studies will be discussed her.

1.1.1 The comet assay

Although the alkaline comet assay (single cell gel electophoresis) was primarily developed as a method to measure DNA damage, it has also been used to measure DNA repair. The standard version of the comet assay measures DNA strand breaks (SBs) in individual cells. The protocol is simple [6]: briefly, cells are embedded in agarose, placed on a microscope slide and lysed to remove membranes and soluble components (including histones) leaving nucleoids (i.e., supercoiled DNA attached at intervals to a nuclear matrix forming loops) [7]. After that, nucleoids are exposed to an alkaline treatment and to alkaline electrophoresis. The presence of breaks in the DNA relaxes the supercoiled loops and enables the DNA to migrate towards the anode. Finally, DNA is stained with a DNA fluorescent dye and visualized by fluorescence microscopy, revealing images similar to the stellar comets. The more breaks that are present, the more DNA is able to migrate to the anode. The percentage of DNA in the comet tail represents the frequency of DNA SBs and is measured by image analysis. It is worth to mention that DNA cross-links have the opposite effect; they inhibit the migration of the DNA loops. About 50-150 cells (comets) are evaluated per sample and the mean or median value is normally calculated as the descriptor of the sample. Visual scoring methods have been used, though it is currently not the method of choice. In this system, comets are visually classified in 5 categories according to the intensity of the comet tail and head [8]. Each comet is given a value between 0 and 4; 0 for undamaged comets and 4 for the comets with almost all DNA in their tail. The overall score is calculated by applying the following formula: (percentage of cells in class 0 x 0) + (percentage of cells in class 1 x 1) + (percentage of cells in class 2 x 2) + (percentage of cells in class 3 x 3) + (percentage of cells in class 4 x 4). Consequently, the total score is in the range from 0 to 400 arbitrary units (AU). This system gives reliable results when applied by an experienced operator and is comparable to the scores obtained using image analysis systems [9].

The digestion of the nucleoids (i.e., naked DNA remaining after the lysis of the cells) with lesion specific enzymes allows the detection of other lesions such oxidises bases [10]. Formamidopyrimidine DNA glycosylase (Fpg) is the most used in order to detect 8-oxo-7,8-dihydroguanine (8-oxoGua) though it also detects other DNA lesions.

1.2 Comet based approaches to measure DNA repair

1.2.1 Cellular repair assays

The most straightforward approach to measure DNA repair activity is to induce DNA damage in cells and subsequently monitor the rate of repair/removal of these lesions over time.

Interestingly, the comet assay was developed to measure DNA repair from the very beginning; followed the reduction in the number of radiation-induced breaks with time, which represents the repair of those lesions [11]. Singh et al., used what is now referred to as a 'challenge assay' or 'cellular repair assay' (as it will be called in the rest of this paper), which follows the kinetics of removal of a certain DNA lesion and re-ligation of the remaining SB with time (Figure 1) [11].

SB re-ligation following X- or ɣ-irradiation, or H_2O_2 treatment has been extensively studied in human biomonitoring [12], but it is possible to monitor the removal of other DNA lesions such as oxidised and alkylated bases, and UV-induced cyclobutane pyrimidine dimers, using appropriate enzymes to convert the lesions to SBs [6]. The specificity of the cellular repair assay, regarding the DNA repair pathway that is measured, depends on the DNA-damaging agent, the version of the comet assay (i.e., with or without enzymes) and the substrate specificity of the enzyme used.

The advantage of this assay is that the entire DNA repair process is assessed, since it depends on the restoration of the normal DNA structure. Moreover, since DNA repair is measured at a cellular level, the presence of cell populations with different DNA repair capacity can in theory be detected. However, from a technical point of view, it is rather complicated to measure repair in this way, because it requires hours of cell culture and sampling at intervals for comet assay analysis, highly limiting the number of samples that can be analysed at the same time.

The fact that cells (normally white blood cells) are under *ex vivo* conditions might also influence the DNA repair process. Although there is no direct evidence to support this notion, the higher *ex vivo* oxygen tension, compared to the *in vivo* conditions, could for instance alter the repair process. Moreover, interpretation of the results is complicated by the fact that DNA-damaging agents may induce different amounts of lesions in different subjects, so that repair starts at different substrate concentrations [12]. This may be particularly important if the initial amount of damage is too high, reaching the point of saturation of the comet assay.

1.2.2 Inhibitor-based cellular repair assay

DNA repair capacity can also be measured by including polymerase inhibitors such as aphidicolin or cytosine arabinoside in the cellular repair assay; in this way, removal of the affected nucleotide occurs, but the re-synthesis step to fill the gap in DNA is inhibited [13-15]. As a result, the normally transient SBs accumulate to an extent which reflects the repair capacity of the cells. Although from a technical point of view this assay is simple, its application in human biomonitoring studies is very rare. The assay has been successfully used to assess

NER capacity [14, 15], but it is worth mentioning that some authors have reported that the DNA breaks produced during NER are not necessarily transient in freshly isolated lymphocytes and are detectable with the comet assay without using additional polymerase inhibitors [16, 17]. In any case, the use of polymerase inhibitors may increase the sensitivity of the assay by increasing the %DNA in the tail and avoids misinterpretation of the results (e.g. inter-individual differences resulting from different precursor pool sizes rather than actual differences in repair). The application of this method to biomonitoring requires further investigation and validation.

1.2.3 *In vitro* DNA repair assays

As an alternative to assessing repair carried out by intact cells, a more biochemical approach - referred to as *in vitro* DNA repair assay- has been developed. This approach is based on the capability of repair proteins in a cell extract to recognize and incise substrate DNA that contains specific lesions. The whole-cell extract can be prepared from lymphocytes, ground tissues or cultured cells, by 'snap-freezing' and subsequent lysis with Triton X-100. At the moment, there are distinct types of *in vitro* DNA repair assay.

One of these approaches uses closed circular plasmids containing specific lesions as substrate. When incubated with the cell extract, repair enzymes within this extract can incise the plasmid close to the lesion and the resulting nicked (repaired) or closed (unrepaired) plasmids can be separated by gel electrophoresis [18]. In an alternative version of this plasmid assay, the cell extract is incubated with the plasmid in the presence of ^{32}P-labelled deoxyribonucleoside triphosphates and repair can be estimated by the incorporation of radioactive precursors into a repair patch [19]. In this way the plasmid assay measures the overall repair starting from incision to repair synthesis. Alternatively, the cell extract can be incubated with an oligonucleotide that is constructed with a specific DNA lesion and a terminal radioactive or fluorescent tag [20, 21]. The repair enzymes in the extract will cut the oligonucleotide at the damaged site, causing the release of the label or a change in the size of the single stranded oligonucleotide, which can be measured as an indicator for DNA repair.

Although these methods have been applied in human biomonitoring studies, especially by Paz-Elizur *et al*. ([22-24]) and Leitner-Dagan, *et al*. ([25, 26]), the number of studies in which these techniques were applied is limited. The comet assay on the other hand has been used as an *in vitro* DNA repair assay more often and its principle plus multiple applications has recently been reviewed [3, 10]. The DNA incision activity of a cellular extract is measured by incubating

it with agarose-embedded nucleoids containing specific lesions as substrate (nucleoids can be derived from established cell lines or white blood cells) (Figure 2). The DNA repair enzymes in the cell extracts will recognize the damage in the substrate nucleoids and induce repair incisions. The comet assay reveals the incision activity of the enzymes by the accumulation of breaks in the substrate nucleoids. (It seems that the pool of deoxynucleotide triphosphates (dNTPs) in peripheral blood mononuclear cells (PBMC) is so limited and diluted that the re-synthesis stage of DNA repair cannot take place. When dNTPs are added to the cellular extract, DNA synthesis and ligation occurs and breaks/incisions are no longer detected [27].

Thus, the nature of the DNA lesions in the substrate defines the type of DNA repair that is measured. BER and NER have been extensively studied using this approach [28]. In the case of the *in vitro* BER assay, substrate nucleoids are commonly produced by treating cells with the photosensitiser Ro 19-8022 and subsequent irradiation with visible light or cells are treated with potassium bromate to produce 8-oxoguanine (8-oxoG) [29, 30]. For the *in vitro* NER assay, substrate nucleoids are produced by treating cells with benzo[a]pyrene-diol epoxide (BPDE) to induce bulky adducts [31], with UV(C) to induce pyrimidine dimers [32], or with oxiplatin to induce cross-links [33]. In these *in vitro* assays, the incision activity is considered to be the rate-limiting step of the DNA repair process, and is measured as an indicator of the DNA repair activity. This method is more convenient for human biomonitoring studies than the cellular assay, since several samples can be easily analysed at the same time and it can be performed with frozen samples [34]. Most of the publications using the *in vitro* repair assay to measure DNA repair activity in humans use lymphocytes or PBMCs. There are few human studies using tissue samples; to the best of our knowledge only colon has been analysed [33, 35, 36].

2. Technical validation and optimizations

The cellular repair comet assay and the *in vitro* repair comet assay for BER and NER have been extensively used in assessing DNA repair for biomonitoring purposes. However, although several protocols regarding the different approaches have been published, most laboratories use their own protocols, which leads to significant variations in procedures and potential difficulties in carrying out inter-laboratory comparisons of results. In fact, all techniques used in molecular epidemiology should be validated before routine use, so that there can be confidence in the results, and comparability between laboratories and studies.

2.1. Cellular repair assay – optimizations & lack of validation

Protocols to carry out the cellular repair assay, covering the measurement of repair of SBs, oxidised bases (BER) and UV-induced pyrimidine dimers (NER), were published by Collins and Azqueta [37]. As mentioned in the introduction, this is a simple but tedious approach. According to our knowledge, though the approach has been extensively used, validation studies have not been carried out (or published) and there are still some pending technical issues. Foremost, the effect of the type of DNA-damaging agent on DNA repair activity has not been tested (*e.g*. X-, ɣ-irradiation, *vs*. H_2O_2 to induce SBs; different photosensitisers plus light *vs*. potassium bromate to induce oxidised bases; UVC-light *vs*. BPDE to induce lesions repaired by NER). Additionally, analysis of results is an issue since individuals can vary in the level of damage induced experimentally in the test cells, due to differing individual susceptibility to the DNA-damaging agent (e.g. varying antioxidant status leading to different levels of base oxidation). Therefore, the use of t½ (*i.e*. the period of time at which half of the DNA damage has been repaired) could be a good option in order to compare results among different individuals, assuming first order kinetics, because t½ may be independent of the initial amount of damage [37].

The approach of measuring DNA repair by blocking polymerase and re-ligation after incision using DNA polymerase inhibitors has been described as a potential tool to be used in human biomonitoring studies, but it has not yet been applied in large scale studies [15, 38]. It presents the same unsolved technical issues as the cellular repair assay.

2.2. The validity of the *in vitro* repair assay

Collins and Azqueta described the practical details for applying the *in vitro* repair assay [37] and a detailed protocol to carry out this assay in cultured cell lines, blood cells, animal tissues and human biopsies, was published in 2013 [34]. The protocol includes practical tips and recommendation for setting up the assays. This is the most convenient adaptation of the comet assay to measure DNA repair in human biomonitoring studies and several technical validations have been carried out.

The usefulness of the *in vitro* BER assay was demonstrated several times since the very first paper in which the approach was described, measuring the repair activity of extracts from cells/tissues of OGG1 knockout cells and mice in comparison to wild type (WT) material. In all cases the activity decreased or completely disappeared in knockout samples [29, 36, 39, 40]. The usefulness of the *in vitro* repair assay to assess NER activity was demonstrated by Langie *et*

al. using extracts of cell lines established from patients with xeroderma pigmentosum (XPA-/-, XPC-/-) and WT fibroblasts [31]. They found lower DNA incision activity when extracts from the knockout cells were used and, as expected, the activity was restored to normal WT values when mixing the extracts of XPC and XPA mutants, because they complement each other. Slyskova *et al*. measured the NER repair activity of extract from liver of XPG-/- and WT mice, showing that knockout mice had no more activity than the negative control incubations with reaction buffer alone [36].

The protein concentration of extracts can be measured and concentration adjusted, though Collins *et al*. reckoned that determining the extract concentration on basis of the cell numbers is sufficiently accurate when using lymphocytes [29]. However, in some cases cells are lost during centrifugation; and the extraction efficiency of proteins can differ slightly between batches. Therefore, it is recommended that the concentration of proteins should be measured in each extract [34]. In the case of extracts from tissues, the protein estimation is essential [40, 41], since tissue samples consist of an unknown number of cells, containing a mixture of cell types and connective tissue.

The incision activity at different extract protein concentrations normally shows a non-linear relation or a linear but not proportional relationship between incision and concentration. Collins *et al*., showed a linear but not proportional relationship of extract concentration and BER activity when 0.25X, 0.5X and 1X extract was used [29]. Guarnieri *et al*. also found a linear but non-proportional relationship when testing the BER activity of different mouse liver extract concentrations (0.001, 0.01, 0.1 and 1x) [39]. In an experiment testing different concentrations of extract from human colon biopsies (0-18 mg protein/ml), a non-linear relationship was reported: a non-proportional increase in activity was seen until 3 mg/mL followed by a decrease at higher concentrations [36]. The authors explained that too high protein concentration saturated the reaction. In the same study, similar effects were observed when the NER incision activity was measured. Likewise, when testing human lymphocytes or cultured fibroblast for their NER activity; high protein concentrations caused a lower relative difference between the total damage-related incision activity and non-specific incisions [31]. Therefore, when working with tissues, extract dilution curves should be performed to elucidate the protein concentration showing the maximum activity, since important differences in the optimal concentration among tissues (especially between proliferative and non-proliferative tissues) have been shown in animals [40, 41]. This probably also applies when using human tissues.

Heat inactivation of extracts from animal tissues demonstrated that the SBs in substrate DNA are produced by enzymes contained in the extract and so the assay is measuring enzyme activity [40-42]. Slyskova *et al*. used aphidicolin or ABT888, inhibitors of the post-incision (repair synthesis) phase of BER and NER respectively, to check if they could increase the specificity of the assay and prevent underestimation of the detected incision activity of the protein extracts (from human colon biopsies) [36]. Incision activity could be underestimated due to the presence of some level of repair synthesis occurring. However, if that were the case, the inhibitors would have enhanced the yield of breaks, but this was not observed [36].

The lack of non-specific nucleases in extracts from lymphocytes has been demonstrated by the low level of SBs present in untreated substrates [29]. However, significant non-specific nuclease activity was detected in extracts from animal tissues [40, 41]. In this case, altering the reaction buffer was used as strategy to decrease the non-specific enzyme activity (adding proteinase inhibitors, ATP, polyAT) [40, 41]. Although these changes decreased the non-specific nuclease activity, a simultaneous decrease in repair-specific incision activity was observed. Interestingly, aphidicolin may have an inhibitory effect on various nucleases that are not related to DNA repair processes. For instance it had been demonstrated to inhibit Herpes Simplex virus DNA polymerase-associated nuclease activity [43], as well as the 3'→5' exonuclease activity of eukaryotic polymerases δ and ε [44, 45]. Only the use of aphidicolin significantly increased the specific incision activity of mouse liver and brain extracts by decreasing the non-specific endonuclease activity in the BER assay [40], but did not have such an effect in mouse colon and lung [41]. To reduce non-specific incision activity in mouse colon and lung the protein concentration of extracts had to be decreased or additional washes during extraction had to be performed [41].

The reproducibility or inter-experimental variability of the assay has also been demonstrated for different types of samples (lymphocytes and colon biopsies) for both the *in vitro* BER and *in vitro* NER assay by analysing duplicate samples on different days [29, 31, 32, 36]. This indicates that the repair activity is stable after storage of samples. Similarly, long-term preservation of animal tissues and extracts to be used in the *in vitro* DNA repair assays has been demonstrated for BER [40]. Regarding the NER assay, the situation depends on the storage of the sample; either as cell pellet or protein extract, plus the addition of ATP to the extract. The use of ATP or an ATP-regenerating system in the extract is not needed when the BER assay is carried out in lymphocytes [29]. When assessing NER incision activity similar results have been obtained with or without adding ATP to freshly prepared cell extracts [31, 32], demonstrating that samples contain enough ATP to carry out the first reactions of the repair process. However, Langie *et*

al. showed that protein extracts lose their activity after long-term storage (i.e. several weeks) at -80°C and that activity is restored by adding ATP [31]. Cell pellets stored at -20°C kept their activity for at least 40 days and the addition of ATP did not increase activity [31]. Some authors claim that magnesium is essential for the detection of NER activity [32]. However, it is advisable to test this for each new cell type or tissue under study, as a too high magnesium concentration in the extract could enhance non-specific nuclease activity, as demonstrated in the BER assay when using mouse tissue extracts [40, 41].

2.3 Crucial parameters to consider

The incubation time of the extract with the substrate is a critical parameter of the assay; time-course experiments showed an initial linear increase in SBs followed by a plateau [29]. The optimal time of incubation should be selected from the linear part of the curve, but showing a high enough BER or NER incision activity. Several incubation times have been reported, many of them based on preliminary studies [31, 39, 40]. These variations in incubation times could be partially due to the different adopted incubation methods; some researchers use humid boxes placed in an incubator, while others use a 'slide moat'. It is crucial to select an incubation time which detects enzyme or extract activity in the linear phase of the titration curve, not to reach the plateau.

Langie *et al*., studied the effect of varying the agarose concentration in the BER assay; the agarose concentration may affect the penetration of the enzyme and in consequence the incision repair activity of, in this case, mouse liver extracts [40]. Indeed, an inter-laboratory comparison was published in 2013 [46], in which the incubation step of the nucleoids with the repair extract seemed to be an important stage in the protocol that led to large inter-laboratory variation. In this trial, 8 laboratories tested the BER activity of three cell lines starting with cell pellets or with cell extracts, both provided by the coordinating laboratory. The 6 most experienced laboratories reported the same cell line as having the highest activity. A significant correlation was reported between the repair activity found when testing the provided extract and the self-made extract from the provided cell pellet; this suggests that the predominant source for inter-laboratory variation was the incubation of the extract with the substrate. Though detailed instructions were given to prepare the cell extract or to assess the repair activity of the provided and self-made extracts, each laboratory used their own conditions for the comet assay. Therefore, more attention should be given to standardize this

401 particular steps (i.e., agarose concentration and extract incubation) and the penetration of
402 repair enzymes into the gel.

403

404 **2.4. Outstanding issues that warrant further technical investigation**

405 In the protocol published by Azqueta *et al*., some outstanding technical issues were noted [34].
406 These and additional technical issues are outlined below:

407 1) DNA incision activity can be studied in relation to the number of cells in the extract, the
408 protein concentration or the DNA content, but the accuracy of the different options has not
409 been studied.

410 2) Although, aphidicolin is mainly known as a DNA polymerase inhibitor, the use of aphidicolin
411 in cell extracts also prevents the occurrence of non-specific nuclease activity in the BER assay
412 [40]. Aphidicolin was described to have an inhibitory effect on various nucleases that do not
413 have a specific role in DNA repair processes. However, its effect when the NER assay is carried
414 out has only been tested once with human colon biopsies [36]. In some cases, it may enhance
415 the detection of NER activity by preventing repair synthesis [40].

416 3) There is a lack of proportionality between repair activity and protein concentration, which
417 needs to be further investigated. Meanwhile, it is recommended that as far as possible extracts
418 should be made from the same number of cells or the same wet weight of tissue, and resulting
419 protein concentrations should be checked.

420 4) The *in vitro* repair assay needs to be validated by comparison with other *in vitro* assays.
421 Some efforts have been made in this direction as is stated in the next section.

422 5) A new 'ring study' involving several laboratories, standard cell extracts and standard
423 protocols should be carried out.

424 6) The most widely used substrate for the *in vitro* repair assay has been Ro19-8022 + light.
425 However, potassium bromate is an easier and cheaper chemical to use. This substrate has
426 been used for repair activity in cell cultures [47] and human biomonitoring studies [30].
427 Interestingly, potassium bromate generates equally high levels of DNA lesions detectable in
428 the hOGG1- and Fpg-modified comet assay, whereas Ro19-8022 + light seems to generate
429 lower levels of hOGG1-sensitive sites as compared to Fpg-sensitive sites [48]. This discrepancy
430 remains to be investigated.

431 7) For the NER assay, both UV light and BPDE have been used to produce substrate nucleoids,
432 and the relationship between the two has not been properly studied; cyclobutane pyrimidine

dimers and bulky DNA adducts are not necessarily recognised in the same way by repair enzymes.

8) It has not been studied so far whether the use of different cell types to produce the substrates (e.g. different established cell lines or human lymphocytes) has any influence on the measurement of DNA repair.

9) There is a lack of true positive controls: i.e., compound that increases the cellular repair for the cellular repair assay, or extracts with a high repair activity for the *in vitro* repair assay. This may be a complicated issue since the induction/modulation of the DNA repair may depends on the cells line/tissue under study. However, some attention should be given to this point. Over recent years, different versions of the alkaline comet assay have been developed in order to increase the throughput. For example, a medium throughput comet assay has been successfully used in an updated version of the *in vitro* BER and *in vitro* NER repair assays, using 12 minigels on microscope slides [34, 36, 49], or 8 deposits on GelBond® films for the Aphidicolin-block cellular repair assay [50, 51]. More recently, further adaptations enable high throughput performance of the comet assay. For example, the use of larger Gelbond® films and reduction of the volume of agarose deposited offer the possibility to increase to 96 minigels processed on the same support [52], but this method has not yet been applied to the repair assays. Other technologies derived from the comet assay, using high throughput microarray or microfluidic approaches, have been proposed to study DNA damage, for example CometChip [53], Microfluidic Comet Array [54] and HaloChip [55]. These techniques have been applied to the cellular repair assay, but to date they are not applicable to the *in vitro* repair assay, either for NER or for BER.

High throughput is crucial for human biomonitoring to allow the processing of a high number of samples. A new challenge is to adapt either the high throughput comet assay or one of the newer derived technologies in order to make it useful not only for the cellular repair assay but also for the *in vitro* DNA repair assays.

3. Comparison of techniques and comparison BER/NER

Comparing techniques with each other, preferably comparing a newly developed assay with a gold standard, is a crucial aspect of the validation of a technique, because it provides information about the extent to which the method actually measures the intended outcome (in this case DNA repair activity). Several studies have performed various assays in parallel, but

the correlations between the outputs of these assays are rarely described. In this section, we describe the various comparisons that have been investigated to date.

A few reports compared data from the comet-based cellular repair assay against plasmid-based repair assays to study BER. Astley et al. (2002) observed an increase in the removal of H_2O_2-induced SBs in carotenoid-supplemented Molt-17 cells by the cellular repair assay, but were unable to confirm these data by means of DNA repair patch plasmid synthesis assays [56].

Incubation of H_2O_2-treated HeLa and Caco-2 cells with β-cryptoxanthin, a common carotenoid, led to a ~2-fold increase in the rate of removal of oxidised purines by BER in the cellular repair assay. This effect was confirmed with the *in vitro* BER assay; incision activity was about twice as high with the extract prepared from carotenoid pre-incubated cells [57]. Ramos *et al*. (2010) showed that water extracts from the *Salvia* species *Salvia officinalis* and *Salvia fruticose*, and the polyphenolic compound luteolin-7-glucoside increased the rate of H_2O_2-induced DNA SB removal in Caco-2 cells [58]. Similarly, pre-incubation for 24 h with extracts of *Salvia Officinalis* and luteolin-7-glucoside increased BER-related incision activity in Caco-2 cells. The same group observed the triterpenoid ursolic acid and the flavonoid luteolin (two compounds present in fruits and vegetables) to enhance the H_2O_2-induced SBs removal rate and BER-related incision activity in pre-treated Caco-2 cells [59].

Although BER has been studied the most, several studies also use the *in vitro* DNA repair assay to study NER in humans [31, 32, 35, 36, 60-62], as well as in cell lines [63, 64], and in animal models [65, 66]. However, as far as we know, only one study reported a correlation of the *in vitro* NER assay with another functional DNA repair method, *i.e*. BPDE–DNA adduct removal over 48 hours as determined by ^{32}P-post-labelling [31]. The slopes of the BPDE-DNA adduct removal curves, were plotted against the DNA incision activity values as measured by the *in vitro* NER assay on substrates containing BPDE-DNA lesions, and showed a significant positive correlation between the two assays (linear regression: R^2=0.76).

Although NER can act as a back-up mechanism for BER in situations of massive oxidative stress paired with high levels of damaged DNA [67-69], these two repair mechanisms are not always affected in the same way by external factors or disease conditions. In a study of seventy patients with sporadic colorectal cancer, BER and NER activities showed a significant positive correlation in healthy colon epithelium (Pearson test: R=0.32) [35]. In contrast, Gaivao *et al*. (2009) did not observe a statistically significant correlation between BER and NER activity in lymphocytes of healthy volunteers [32]. Still, a direct comparison of NER and BER activity is not

498 necessarily informative, because they recognize and repair different types of DNA lesions. In
499 some cases, NER and BER can even be modulated in opposite directions. For instance, Brevik *et*
500 *al*. (2011) observed that BER and NER activities were affected in the opposite way by kiwi fruit
501 and phytochemical consumption (i.e. high intake of a variety of antioxidant-rich plant
502 products) [62]. In addition, storage of blood samples at room temperature for 24h reduced
503 NER activity as assessed by the aphidicolin-block cellular repair assay for NER compared to
504 fresh samples, whereas OGG1 activity (representing BER) was higher after 24h storage at room
505 temperature *versus* freshly isolated samples [50].

506 Overall, both the cellular repair assay and *in vitro* repair assays have proven to be useful and
507 sensitive for studying the modulation of DNA repair by nutritional factors, environmental
508 exposures and disease state (also see section 4). We are convinced that new comet-based
509 repair assays to study additional repair pathways are bound to come in the near future. It will
510 be of utmost importance to include comparisons with available functional DNA repair assays
511 into their validation process.

512

513 **4. Inter- and intra-individual variation in DNA repair activity**

514 Variations in DNA repair activity at the level of the individual are poorly investigated. However,
515 it is important to understand the sources of variation. There is currently insufficient knowledge
516 to conclude to what extent the repair activity of an individual is determined by genetics, or
517 whether it can be influenced by environmental factors. Moreover, variation between
518 individuals in both BER and NER activities cannot be explained.

519 Gaivao *et al.* measured DNA repair activity on several occasions in the course of a nutritional
520 intervention study, involving 30 healthy subjects [32]. Both BER and NER were assessed by
521 applying the *in vitro* repair assay. As the intervention appeared to have no effect on the DNA
522 repair activity, data from the six blood samplings - at 4-week intervals – were used to examine
523 both inter- and intra-individual variation. In Table 2, the correlation coefficients for all
524 timepoint comparisons are shown, for both BER and NER separately. In 9 of the 15
525 comparisons of BER rates, the correlation was statistically significant, and this was true for 12
526 out of 15 comparisons of NER rates. It is interesting that the correlation coefficients did not
527 decrease as the time between samplings increased. Thus, although there may be unknown
528 factors that affect repair activity from time to time, there is an underlying consistency, in both
529 BER and NER, for a given individual. While there was considerable inter-individual variation in
530 both BER and NER activity between subjects (coefficients of variation: 32% and 59%

respectively), the range between highest and lowest activity was substantially higher for NER. Figure 3 shows, as examples, two of the timepoint comparisons for BER and NER. Although it illustrates the relative consistancy of repair rates for individuals, the figure also shows the variety of repair rates between individuals. For BER, most subjects have rates within a 3-fold range; for NER (using UV-exposed substrates in the repair assay), the range is about 7-fold. This is in line with a previously reported 10-fold difference in NER activity using BPDE-exposed substrates in the repair assay [31]. Interestingly both studies [31, 32], reported that some individuals seem to have negligible repair activity. Whether this has any health implications is unclear. It is possible that a low NER rate indicates a reduced intrinsic capacity to deal with UV-induced cyclobutane pyrimidine dimers or bulky adducts, or it could be that individuals with low measured NER activities are not exposed to DNA damage and therefore their repair enzymes are simply not induced.

Similarly, in a group of 122 subjects (mean age 24.5 y, range 19-48 y, 39 men and 83 women), inter-individual variation in NER activity assessed by the aphidicolin-block cellular repair assay in response to BPDE, ranged from 0.66 to 26.14 %DNA in tail (mean 7.38 +/- 4.99 %), showing an almost 40-fold difference across the group [51].

There are some other publications comparing repair rates between individuals using different techniques (Table 3). These studies highlight considerable inter-individual variability in the capacity to repair DNA. Certain factors, such as age and sex, might affect repair activity and recognising such factors would be necessary for the design of human studies and interpretation of repair data from such a trial. The following sections therefore describe which factors, whether host factors (*e.g.*, age, sex and genetic polymorphisms in DNA repair genes) or environmental/lifestyle factors (*i.e.*, smoking, status, diet and health status), may contribute significantly to this variability.

4.1. Host factors

Age and sex:

Numerous studies have reported a strong positive link between increasing age, DNA damage and defective repair [40, 41, 74-81]. However, to date few human biomonitoring studies using the comet assay have established the relationship between aging and repair activity (table 4).

In one study of 375 participants with occupational exposure to asbestos, stone wool and glass fibre, increasing age was associated with increasing DNA BER activity measured by the *in vitro* DNA repair assay using Ro19-8022 with light to induce damage in substrate cells (Correlation

564 coefficient R=0.1) [69]. In contrast, in a study of 244 men and women (mean age 41.3), neither
565 BER of oxidative damage (*in vitro* repair assay) nor irradiation-induced repair (cellular comet
566 assay) was affected by either age or sex [83]. A cross-sectional study of subjects from Denmark
567 showed an inverse association between age (18 to 83 years) and BER activity (using $KBrO_3$
568 treated cells as substrate) in PBMCs; the effect was stronger in women as compared to men
569 [30]. However, in a study specifically designed to assess the impact of age on DNA repair
570 activity, Humphreys *et al*., investigated the relationship between age and BER activity
571 measured by the *in vitro* repair comet assay using Ro19-8022 + light damaged substrate cells
572 [82]. BER was investigated in 3 groups of subjects of increasing age [20-35 y (n-40), 63-70 y
573 (n=35) and 75-82 y (n=22)]. Here, the authors found a positive but weak correlation between
574 age and BER rate (r=0.25). However, it should be mentioned that the authors of this paper
575 state that the inclusion criteria were "relaxed" for the oldest group. Consequently, subjects
576 with disease in the oldest group might have biased the results. DNA repair activity was the
577 same in both sexes.

578 The relationship between age and DNA repair may be further complicated by differences in
579 repair activity in different strata of population studies defined by sex or race. Trzeciak et al.
580 (2008) used a cellular repair assay to study the impact of these factors on repair of γ-radiation-
581 induced DNA damage in PBMCs from four age-matched groups of male and female whites and
582 African-Americans between ages 30 and 64 [84]. They reported a positive association between
583 repair activity and age in white females, but a statistically non-significant decrease in African-
584 American females.

585 Overall, the available data suggest that, while sex is not a major contributor to inter-individual
586 variation in repair activity, age is a factor that should be taken into account (for example, by
587 ensuring a similar age distribution in control and test groups) - though as yet there is no
588 indication of a major positive or negative effect. Also animal studies have reported conflicting
589 results. There are recent reports that the effect of age on BER activity (*in vitro* repair assay) can
590 be tissue dependent and that the brain seems to be the most vulnerable for a decline in BER
591 activity with age [40-42, 74]. Future human biomonitoring studies should consider studying
592 DNA repair in other available tissues with different cell turnover, in comparison with blood
593 cells (*e.g*. buccal cells, saliva, colon biopsies, etc.). The effect of race on repair activity and its
594 interaction with age is unclear.

595

596 *Genetics (polymorphisms in DNA repair genes)*

Data from human biomonitoring studies, using the comet assay to assess the associations between genetic variations in DNA repair genes and repair activity are scarce. Vodicka *et al*. performed a relatively large-scale study [244 healthy subjects, 183 men and 61 women, mean age 41 ± 11 y], specifically designed to investigate the impact of various genotypes (XRCC1, APE1, hOGG1, XPD, XPG, XPC, XRCC3 and NBS1) on NER and BER activities. BER (*in vitro* DNA repair assay) was significantly lower in people homozygous for the GG variant of hOGG1 compared with carriers of the normal genotype [83]. The ability to repair γ-irradiation damage (cellular repair assay) was significantly lower in individuals homozygous for the XRCC1 AG genotype. However, in a study by Jensen *et al*., healthy subjects did not show any difference in BER activity (*in vitro* repair assay using Ro19-8022 + light as substrate) associated with the hOGG1 Ser326Cys polymorphism; 49 subjects of each genotype were selected and group-matched from a cross-sectional study of 1019 subjects [85]. Interestingly, there are indications for an interplay between BER and NER, or NER playing a role as a back-up mechanism for BER. For instance, a study on occupational exposure to potential genotoxic agents, observed BER activity (using the *in vitro* DNA repair assay) to be significantly higher in subjects carrying the XPA AA normal genotype compared to the AG and GG variants [69].

In addition a few studies have investigated the gene-environment interactions. In a study of 36 volunteers recruited to explore the impact of nutrient/gene interactions on NER activity (*in vitro* DNA repair assay using BPDE-DNA as substrate), subjects were grouped according to genetic polymorphisms in several NER genes (XPA, XPC, ERCC1, ERCC2, ERCC5, ERCC6, and RAD23B; [60]). Here, NER activity was significantly lower in subjects who carried a relatively large number of "low" NER activity alleles. The XPA G23A gene was the strongest predictor for NER activity, with individuals homozygous for the recessive AA variant of the gene demonstrating 3-fold lower repair activity compared to the normal genotype. Interestingly, this same XPA 23A allele was observed to be associated with lower BER activity (*in vitro* DNA repair assay) in colonic tumour tissues, but not in the adjacent healthy tissue [36]. A recent study investigated the impact of genetic polymorphisms on BER repair activity in 43 patients with recurrent depression disorders and 59 controls without disease [86]. The study included 12 polymorphisms in 4 key BER genes (hOGG1, MUTYH, PARP1, and LIG3), which were linked to the cellular repair activity on H_2O_2-induced SBs, but it should be noted that the sample size reported here is small for a study investigating the influence of genotype on disease risk.

4.2 Lifestyle factors

Cigarette smoking

Although, a meta-analysis (evaluating 38 studies) indicated higher levels of DNA damage in smokers versus non-smokers [87], information on the effect of cigarette smoking on DNA repair activity is conflicting. SB re-ligation activity in leukocytes following γ-irradiation (10 Gy) (using the cellular repair assay), was higher in current cigarette smokers (n=17), compared with non or ex-smokers (n=23) [88]. Similarly, SB re-ligation activity following exposure to γ-irradiation (5 Gy) was elevated in smokers (n=80, 1.05 ± 0.81 SSB/10^9 Da) compared with non-smokers (n=134, 0.77 ± 0.62 SSB/10^9 Da) [83]. However, in this study, BER (*in vitro* repair assay) was not affected by smoking. BER measured using the *in vitro* DNA repair assay was significantly lower in poorly nourished male smokers (n=46, mean age 39 y) compared to well-nourished males and females (n=39, mean age 27 y), with mean incision activity 65.9 AU (95% CI 60.4, 70.0) in smokers compared with 86.1 AU (95% CI 76.2, 99.9) in healthy subjects. Moreover, repair data from the cigarette smokers were substantially less variable within the group when compared with the non-smoking participants (range 30-100 AU and 10 -180 AU in the smoking versus the non-smoking subjects respectively). The same authors also studied the effect of smoking in a cohort of workers in a tire plant by performing the cellular repair assay and the *in vitro* BER assay [89]. Higher rates of repair of irradiation-induced DNA damage were detected in smokers versus nonsmokers, but this was not confirmed by the *in vitro* BER assay with Ro19-8022+light generated substrate.

Dietary factors

The comet assay has been used widely in human biomonitoring to assess both the impact of whole foods (e.g. fruits and vegetables) and specific nutrients (phytophenols, antioxidants and folic acid) on genomic instability, particularly the impact of diet on DNA SSBs and altered DNA bases (e.g. oxidative, alkylation and misincorporated uracil). In addition, several studies have described how nutrition modifies DNA repair activity (Table 5).

After the *in vitro* DNA repair assay came into use in 2001, several researchers started performing it in parallel to the cellular repair assay. Cellular extracts from human lymphocytes showed a markedly higher DNA repair incision activity after a single oral dose of 100 mg CoQ10/day for 1 week compared to controls (~3-fold increase in CoQ10 group) as detected by the *in vitro* BER assay [92]. Similarly, the cellular repair assay, studying the removal of Ro 19-8022 + light induced oxidative lesions, detected a statistically significant ~2-fold higher rate of DNA damage removal in CoQ10 supplemented lymphocytes compared to the control group. In

a small randomised cross-over design study, subjects consuming between 1 and 3 kiwi fruits daily for 3 weeks significantly increased *in vitro* BER activity (Ro19-8022+light damaged substrate cells) in PBMCs from male (n=6) and female (n = 8) healthy participants (26-54 y of age) [93]. Volunteers who consumed 3 kiwi fruits each day showed a significantly elevated plasma vitamin C level and substantially enhanced BER activity compared with pre-supplementation levels (>60%). Supplementation also increased the resistance of isolated PBMCs to oxidative damage and was associated with reduced DNA SBs and oxidised base damage (Fpg-sensitive sites). In contrast, there was no correlation between individual BER rates and markers of DNA damage. A significant association between BER activity, assessed by the *in vitro* DNA repair assay (Ro19-8022+ light damaged substrate cells) and antioxidant status was described subsequently, with elevated plasma lutein/zeaxanthin correlating with high BER activity [82]. Supplementation with carotenoids for three weeks, showed enhanced re-ligation of H_2O_2-induced SBs and increased DNA repair patch synthesis activity compared to their initial repair activity before the 3-week intervention [94]. Similarly, supplementing male smokers (n=46, mean age 39) with slow release vitamin C (500 mg/day) and vitamin E (182 mg/day) was found to significantly increase BER (*in vitro* repair assay) by approx. 27% (95% CI 12 – 41%) after 4 weeks [39]. Inter-individual variation in incision activity was generally consistent within this group (range 30-100 AU). In contrast, feeding healthy subjects (n=43 men and women, mean age 27 y) 600 g of fruits and vegetables, or the equivalent levels of antioxidant vitamins and minerals as a supplement for 24 d, did not change BER activity measured by the same group and using the same assay [39]. Inter-individual variability in incision activity was substantial, ranging from less than 10 to more than 180 AU, with a mean of 86.1 AU (95% CI 76.2 - 99.9).

In a more recent study, feeding male smokers (45-75 y) a diet high in antioxidant-rich fruits and vegetables (n=33) or 3 kiwifruits per day (n=33) for 8 weeks significantly increased total antioxidant levels (2-fold), plasma vitamin C, β-carotene and tocopherol, compared to the control group (n=34). Also BER activity was increased 40% (n=23) and 29% (n=25) upon antioxidant-rich fruits/vegetable or kiwi consumption, respectively) (measured using the *in vitro* DNA repair assay) [62]. Surprisingly, NER activity (*in vitro* repair assay and UVC radiation for substrate), was significantly decreased (39% (n=13) and 38% (n=11); upon antioxidant-rich fruits/vegetable or kiwi consumption, respectively). In contrast, feeding young male smokers steamed broccoli (250mg/day for 10 days) did not alter BER activity (*in vitro* repair assay; [91]). A similar lack of effect of antioxidant supplementation on BER (*in vitro* repair assay) has also been described in 48 young healthy volunteers given 100 µg selenium, 450 µg vitamin A, 90 mg

vitamin C and 30 mg vitamin E supplements for 6 weeks [71]. Inter-individual BER activity was substantially different between the volunteers (41-fold). NER (using the *in vitro* repair assay with BPDE-DNA as substrate) was also found to be unaffected by supplementing healthy participants (114 female and 54 male subjects aged between 18 and 45 y) flavonoid-rich blueberry and apple juice (1L/day) for 4 weeks [60]. In this study inter-individual variation, while considerable, was maintained across the two sampling periods (correlation: R=0.69).

While the majority of studies report the impact of food or supplements rich in dietary antioxidants on DNA repair activity, a few studies have investigated the impact of other key dietary agents. Low intake of folate is associated with an increased risk of several human cancers, particularly colon cancer [95]. Numerous studies have reported that folate deficiency induces genomic instability and malignant transformation *in vitro*, in animals and in human studies [95]. In a relatively large-scale, randomised double blind-placebo controlled intervention study, participants (n=61, 20-60 y of age, male and female non-smokers and non-supplement users) were given 1.2 mg folic acid daily for 12 weeks to investigate whether enhancing folate status could improve markers of genomic stability, including BER incision activity measured using the *in vitro* DNA repair assay [90]. BER incision activity was similar across both intervention groups prior to supplementation, with a median value in both treatment groups of 63 AU, extending from 34 and 93 AU (2.5 fold range). While there was no association between red cell folate status and BER activity at the start of the study, increasing folate intake resulted in significantly decreased BER in those volunteers with the lowest pre-intervention folate levels, indicating that BER can be modulated by folate status.

These studies highlight that diet (and supplement use), has a significant influence on DNA repair activity. The impact of other common nutrients and non-nutrients (such as alcohol and caffeine), as well as other lifestyle factors (including physical activity) on inter-individual variation in DNA repair activity measured using the comet assay remains largely unknown and therefore deserves further attention.

Health status

The comet assay has been used widely to determine the relationship between DNA damage (as a marker of genome instability) and various diseases including cancer, vascular disease, diabetes and inflammation. [96] [97] [98] [99]. However, only a few studies to date have investigated the impact of health status (particularly malignant transformation) on NER activity using the comet assay.

Palyvoda *et al*., measured NER repair of γ-radiation-induced (2 Gy) DNA SBs in lymphocytes isolated from 44 healthy donors and 38 patients with squamous cell carcinoma of the head and neck (SCCHN), prior to treatment [100]. The cellular repair assay, following a time course of repair post-irradiation (0-180 min), was used to measure endogenous DNA SBs, radiation-induced damage, rate of repair and residual or non-repaired damage in isolated lymphocytes cultured for 24 h prior to treatment. Endogenous DNA SBs was almost 3-fold higher in patients with SCCHN compared with healthy subjects (median 90.3 vs 33.3 AU respectively), with significantly more individuals in the cancer group showing a high level of damage. Overall, NER repair rates were not significantly different between participants with and without cancer, due to substantial variation in measured repair activity across all individuals. However, by stratifying individuals into subjects with high endogenous DNA SBs, high induced DNA damage, low NER rate and high residual DNA damage, a significantly higher proportion of cancer cases displayed this "negative phenotype" compared with healthy participants (39.4% vs. 7.3% respectively). The variation in DNA damage and repair in this study was substantial, making it difficult to draw strong conclusions. It is also important to note that cases and controls were not matched in this study, and that age, sex and cigarette smoking status were markedly different between the two groups. A significant association between cancer incidence and low NER rate was observed in a smaller study of SCCHN cases (n=12) and healthy donors (n=15), in this case matched for age, sex and cigarette smoking [88]. Whole blood was used to measure DNA damage and repair following γ-irradiation (10Gy) using the cellular comet assay, without pre-culture, and assessing percentage tail DNA using computerised image analysis. Here, DNA repair activity was significantly lower in patients with SCCHN cancers relative to controls (46.5% v 36.8% respectively).

How other human pathologies and effectors of health and disease, such as low-grade sustained inflammation [101], affect individual variation in DNA repair activity is essentially unidentified and represents a substantial gap in knowledge. In any case, the studies so far reported, have been case-control studies and it is not possible to discern whether a difference in repair activity is a cause or an effect of the disease (or possibly an effect of treatment). What is really needed is a prospective study, *i.e.* a large cohort of healthy subjects whose repair activities are measured and who are then followed up for a long enough period of time for disease to develop and be recorded.

4.3. General comment on individual variation in DNA repair

The studies reviewed above show that age, sex, health status, diet, and other lifestyle factors such as smoking, impact to some extent on DNA repair (BER and NER) activity and contribute substantially to the significant inter-individual variation in repair rates described in numerous human studies. It should also be noted that large assay variation may be interpreted wrongly as inter-individual or intra-individual variation. However, if intra-individual variation (estimated from repeat measurements on different occasions) appears to be at a low level, assay variation can be discounted. There is a need for controlled studies that systematically assess inter-, intra- individual and assay variation in for instance ring-trials. One approach would be similar to the ECVAG ring trials on DNA damage endpoints, in which contributors to the overall variation were assessed in a systematic manner [102-105].

Host factors such as age and sex, and certain anthropometric characteristics such as body mass index can be relatively easily dealt with by carefully matching control and test groups. Adjusting for other factors, such as single nucleotide polymorphisms in DNA repair genes is more difficult, principally due to the requirement for substantially larger numbers of participants to adequately power these biomonitoring studies. Genetic variation in DNA repair genes can also be included in intervention studies as effect modifiers [60]. While specific dietary items obviously have an effect on repair activity, as discussed above and reviewed before [106], it is difficult due to lack of information to estimate the impact of other lifestyle factors such as physical activity, sunlight exposure, drug use and health status on inter-individual variation in DNA repair activity. The advantage of studying DNA repair as phenotypic marker rather than single nucleotide polymorphisms or gene expression is that the latter do not take into account epigenetic and post-transcriptional modifications that can affect the final DNA repair activity.

5. Repair activity kinetics and timing

No studies have specifically assessed DNA repair kinetics in a time-course investigation in humans, using multiple sampling over a short period of time. Assessing DNA repair kinetics is important for selecting optimal sampling times relative to exposure. For instance, if exposure to DNA damaging compounds induces DNA repair, a measurement shortly after exposure will indicate higher levels of repair. On the other hand, after a longer period of time when damage has again decreased due to DNA repair or cell death, such an increase in DNA repair activity may no longer be detectable. Unfortunately, most of the information on DNA repair kinetics originates from biomonitoring studies using the *in vitro* DNA repair assay, assessing DNA repair

796 in samples that have been obtained at a single time-point before, during or after a change in
797 exposure.

798 Only one study has investigated the effect of short-term phytochemical supplementation on
799 repair activity. Intake of green tea was associated with increased BER activity in lymphocytes
800 that were obtained 60 and 120 min after drinking 200 mL of freshly prepared tea [107].
801 Although this study suggests that changes in DNA repair activity after a particular exposure can
802 be very quick (minutes to hours), most studies that investigated dietary interventions actually
803 studied the changes over a period of several days to weeks (see paragraph 4.2). Regarding
804 green tea consumption, 12 weeks of regular green tea consumption indeed significantly
805 increased *in vitro* BER activity toward Ro19-8022 + light generated DNA damage in
806 lymphocytes [108], but the study by Ho *et al*. (2014) [107] suggests that this change could
807 already have been detected at much earlier time points. Time points chosen for sampling in
808 other dietary interventions with *in vitro* BER or NER activity as endpoint vary between 1 to 8
809 weeks [92, 62, 71, 93], with reported washout periods between 1 and 2 weeks [92, 93].
810 Interpretation may become more complex if the intervention is performed in smoking
811 individuals, because smoking by itself may already affect BER or NER activity [39, 91] (see
812 paragraph 4.2).

813 It is a matter of debate how the activity of hOGG1 in human cells is regulated, as the *OGG1*
814 gene may be constitutively expressed [28]. Presence of DNA damage seems logical as an
815 inducer of DNA repair. Indeed, animal studies show that DNA repair can be induced by specific
816 DNA damaging triggers and that alterations in repair activity are relatively quick (within days)
817 [65]. In *in vitro* studies with cell lines, induction of BER or NER can occur within hours [58, 59,
818 101, 109]. The number of investigations in which changes in DNA repair were studied after a
819 specific exposure of humans is limited: A study with controlled exposure to wood smoke,
820 although statistically underpowered, showed a slightly increased *in vitro* BER activity and
821 increased urinary 8-oxoGua (*i.e.* repair product of hOGG1) at 20 h post-exposure [110].
822 Another short-term study reported increased levels of oxidatively damaged DNA and unaltered
823 BER activity in PBMCs after 6 or 24 h controlled exposure to traffic-related air pollution [111].
824 Likewise, oral exposure to nanomaterials showed increased levels of oxidatively damaged DNA
825 in the liver of rats at 24 h post-exposure, whereas the *in vitro* BER activity to Ro19-8022 + light
826 generated DNA substrate cells was unaltered [112].

827 To summarise, BER and NER kinetics have not been well investigated in humans and animals.
828 There are inconsistent reports of altered BER activity after dietary interventions and particle
829 exposure, but sampling times are not frequent enough to draw any conclusions on the time

frame in which the changes occur. There are currently too few studies on NER activity to speculate about timing of sampling for assessment of changes in repair activity. From the available literature, it is not possible to suggest an optimal time of sampling in relation to exposure for the assessment of BER and NER activity. Therefore, to improve the applicability of DNA repair measurements in human biomonitoring, it is essential to perform studies in which repair activity is assessed at various time points after exposure/ intervention.

6. Surrogate vs. target tissues

PBMCs (frequently referred to as lymphocytes) are extensively used to measure DNA repair activity in human biomonitoring studies. They circulate through the whole body and are regarded as sentinel cells since they can have a relatively long life-span [113]. Moreover, they are easily obtained, available in large numbers and easy to handle and culture if necessary. The purity of the cells fraction is normally not specified and a mixture is probably the most commonly used material. While they are convenient as surrogate cells, circulating blood cells are not the target for carcinogenesis, and the response of these cell types does not necessarily mimic the effect in true target tissue cells. Also, confounding factors (e.g., smoking, diet, medication, air pollution, exercise) should be taken into account [33, 114], because the reaction of surrogate cells in the exposure-outcome relationship may be different in target organ cells. However, using white blood cells is relatively non-invasive and they are the surrogate cells of choice in studies where (as is usually the case) the target tissue is not readily attainable [115].

There are only 3 studies with humans in which tissues other than lymphocytes or PBMCs have been used to measure DNA repair activity by the comet assay [33, 35, 36]. In these studies, DNA repair activity was measured in colon biopsies and two of these assessed the correlation between DNA repair activity in tissues and PBMCs.

Herrera *et al*. observed that DNA cross-link repair activities of colon tumour epithelial cells and lymphocytes from colon cancer patients (using the *in vitro* repair assay) were not correlated. Thus, lymphocytes were not predictive for the repair ability of the tumour [33]. Slyskova *et al*. found a positive correlation in BER and NER activity between PBMCs and healthy colon tissues, but not between PBMCs and colon tumour tissues [35]. More studies are needed to draw conclusions about the suitability of using lymphocytes or PBMCs to reflect the DNA repair activity of healthy target organs. However, studies in which biopsies of organs from healthy people are included are difficult to perform.

Epithelial cells, as specialized components of many organs, have the potential of being an attractive bio-matrix to evaluate the DNA repair activity of individuals. Examples of possible sources of exfoliated epithelial cells in human biomonitoring studies are presented in Table 6. Unfortunately, although there are many studies that use the comet assay to measure DNA damage in buccal, nasal, tear duct, lens and corneal epithelial cells [116], DNA repair activity has never been explored in these biological matrices using the comet assay. Most of these cell types, while not necessarily target cells for carcinogenesis, have the distinct feature of coming into direct contact with various environmental xenobiotics, and so they should provide useful information on the initial response of cells to exposure. Another characteristic of most of the cell types is that they have a rapid turnover; therefore they would only reflect recent events that affected DNA repair. Future studies are needed, addressing the quality and quantity of exfoliated cells that need to be obtained in order to apply the *in vitro* repair assays. Cell recovery should be high enough to make extracts of sufficient volume and protein concentration to apply to substrate cells. Cell counts may be insufficient for buccal cells obtained by mouth rinsing or cheek scrapings (unpublished data). Cell counts are in theory sufficiently high for epithelial cells in urine [117]. Broncho-alveolar lavages [118] and induced sputum [119] also produce a sufficient number of cells, but these are predominantly leukocytes. It should also be noted that repair activity measurements in lavages from the airways are complicated by the fact that respirable toxicants can induce the influx of cells from the blood and the composition of cells in the broncho-alveolar lavage fluid is dependent on the type and stage of pulmonary inflammation. The applicability of epithelial cells for the repair assays needs to be established, as a large proportion of the exfoliated cells may be dead. For small (needle) biopsies an amount of approx. 5 mg of tissue should be enough to make protein extracts [38].

7. Discussion and conclusion

The comet assay and its modifications to measure DNA repair activity are frequently used in human biomonitoring studies. However, for the correct interpretation of the data of such biomonitoring studies, validation studies are needed that have to date not been performed in a systematic way. In this manuscript, we have compiled the information that is needed for the validation of the DNA repair comet assays, including intra- and inter-Individual variation, repair kinetics, the use of surrogate tissues, and comparison with other methods.

The intra-individual variation over a relatively short period of time (weeks to several months) was reported to be small for both NER and BER, because measurements in the same individuals at two different moments correlated significantly and the slope of the regression line was close to 1.0. This indicates that the measurement of DNA repair activity reflects an individual's intrinsic repair activity.

How a low DNA repair activity should be interpreted is an open question; a person can have a low repair activity and may thus have a higher cancer risk, but it is also possible that low DNA repair activity simply reflects the absence of exposure, and thus DNA repair is not needed. Therefore, for proper interpretation of DNA repair activity data, a combined analysis with exposure data and/or other biomarkers (particularly DNA damage) is required.

It is important to understand the kinetics of DNA repair after exposure. If DNA repair is measured shortly after a DNA damaging exposure, DNA repair may still be induced. On the other hand, when repair activity is assessed at a later time point relative to the exposure, DNA damage may already be removed and repair is no longer needed. Knowledge about the inducibility of DNA repair is therefore indispensable.

The literature is equivocal about the regulation of BER, but NER is likely to be inducible. The different DNA repair pathways are likely to have different modes of regulation. BER often deals with DNA damage induced by endogenously produced DNA reactive compounds. For instance, reactive oxygen species are continuously present (and needed) in the body, but can also lead to oxidised DNA bases. Therefore, these oxidised DNA bases can be considered as physiological DNA lesions and the enzymes involved in BER are thus assumed to be in some way constitutive. In contrast, NER most often deals with damage caused by exogenous agents (i.e., chemicals and radiation), so the enzymes involved in this pathway are probably only synthesized when needed in episodes of increased exposure.

This inducibility of NER may also be reflected in the inter-individual variation that is observed in the general population, because the inter-individual variation in NER is reportedly higher than the variation in BER. The inducibility of NER may be related to lifestyle factors in combination with the genetic background. Surprisingly, some healthy subjects seem to have undetectable levels of NER when using comet assay approaches, which could reflect a lack of exposure or a limitation of the comet assay approach. This observation therefore needs confirmation by using other assays. For interpreting NER data, we therefore suggest that these should always be combined with exposure data.

Although the literature suggests that BER activity is less inducible, some studies showed that dietary interventions may still increase BER activity. Induction of repair activity can, of course, occur post-translationally as well as at the level of transcriptional regulation. Therefore, more work is needed to understand the impact of lifestyle, including genetic background, exposure and dietary habits on both BER and NER activity.

Human biomonitoring studies most of the time use leukocytes or PBMCs to assess DNA repair activity. Only a limited number of studies showed a correlation between DNA repair in PBMCs and the target tissue cells, so more work is needed to confirm that repair in blood cells actually reflects the intrinsic repair capacity of internal organs. However, the work that has been published to date looks promising. The total blood cell population (*i.e.*, leukocytes) consists of different cell types including monocytes, lymphocytes and granulocytes. These cell types have differences in life span, concentrations in blood and most probably also different levels of DNA repair. If common diseases, such as a simple cold, affect blood composition, this could change the repair activity that is measured when using total WBC. In that case, differences in DNA repair activity between or within individuals could be related to the percentages of the different cell types in the blood sample. One should keep in mind that isolating blood cell subpopulations automatically requires more work and hands-on time when preparing the samples and this may not always be feasible in large scale biomonitoring studies. Therefore, a more thorough understanding of DNA repair in blood cell subpopulations may guide the decision to use total white blood cells, isolated PBMCs or PBMC subpopulations in human biomonitoring studies.

It is worth to mention that conflicting results observed in some of the studies summarised in this review can be due to the small sample size. However, these studies often show biologically relevant effects and can give important information for larger future studies. More studies with higher samples size are needed.

However, in order to analyze large numbers of samples in a limited amount of time, as is often the case in human biomonitoring, there is a need to develop high throughput approaches; for instance the CometChip is an approach to be explored [120]. Even if the number of samples per run is increased, samples may still be analysed in batches. To avoid batch differences, the comet assay should be further optimized by, for instance, standardizing the preparation of substrate cells, including positive and negative controls, and using assay controls. Although the COST-Action hCOMET (CA15132) may address some of these issues, it will need concerted action by the comet assay community to carry out a full technical and field validation of the

repair comet assay, to reduce inter-assay and inter-laboratory variations, and to ensure the proper comparison and interpretation of results of biomonitoring studies.

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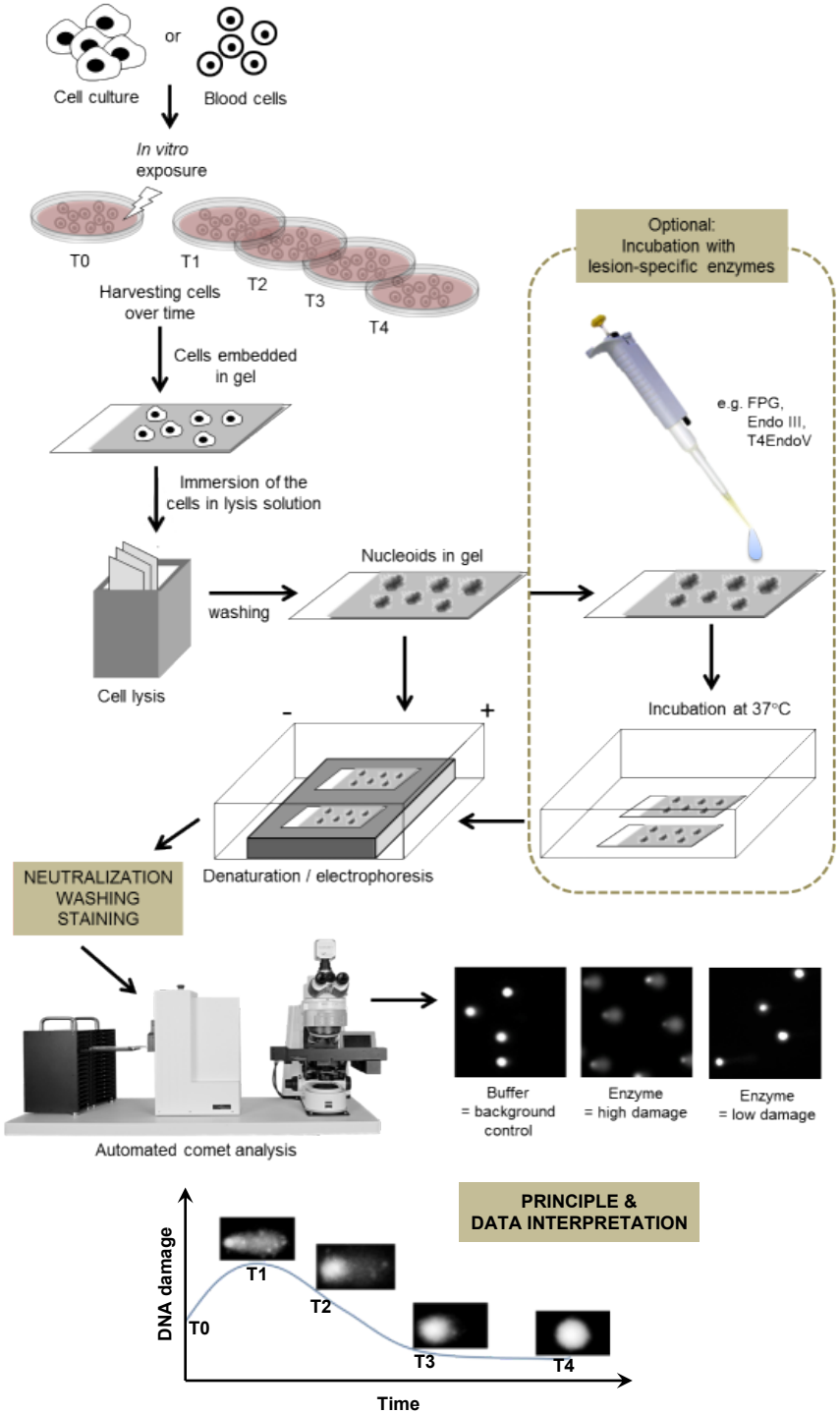
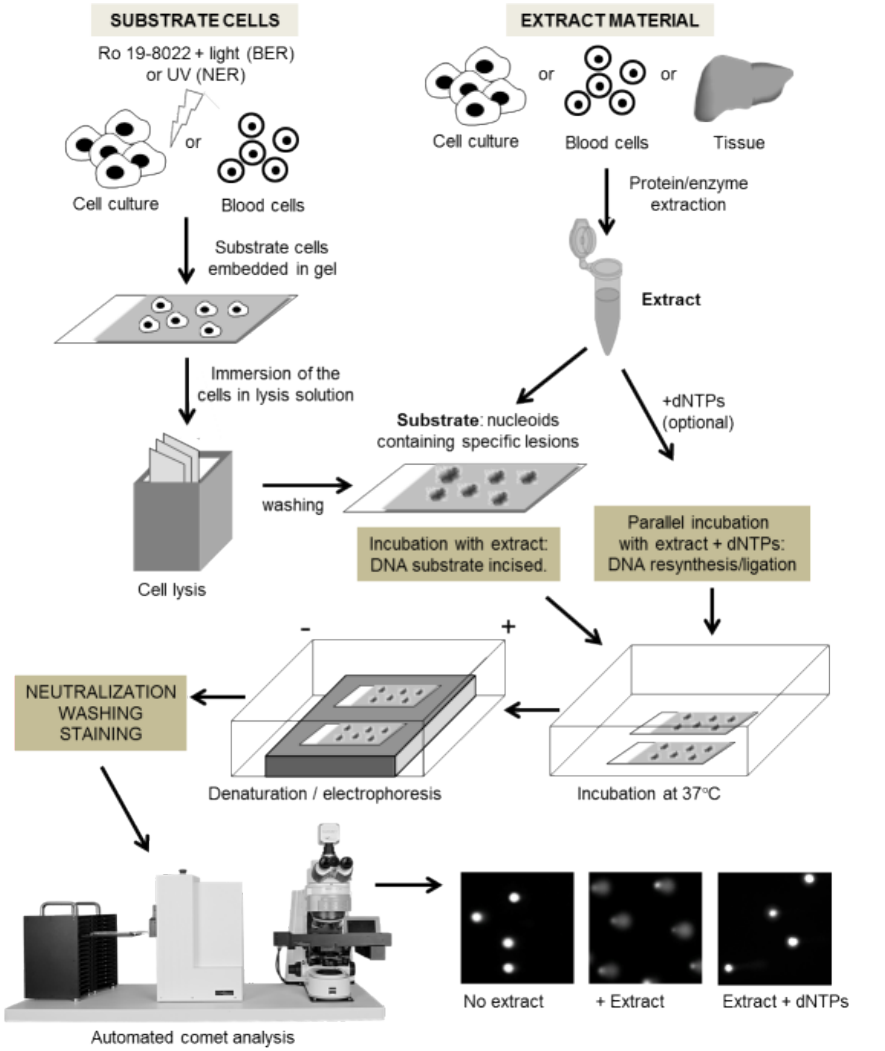
Legends (figures and tables)

Figure 1: Scheme of the cellular repair assay. Nucleoids can either be incubated with lesion-specific enzymes (to assess various specific DNA lesions) or not (to assess SBs). The formation and removal of DNA lesions is studied over time, requiring multiple cell incubations

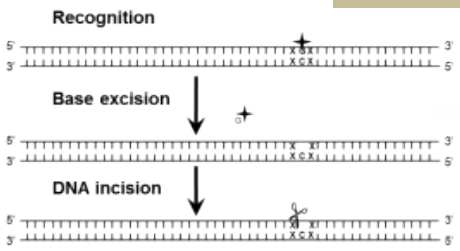
Figure 2: Scheme of the *in vitro* repair assay. Substrate cells can be exposed to the photosensitizer Ro 19-8022 plus light to induce 8-oxodG lesions or to UV to induce primer dimers, allowing the assessment of BER and NER incision activity respectively. After lysis, gel-

1350 embedded nucleoids are incubated with protein extracts for cells in culture, blood or tissues.
1351 Subsequent standard single-cell gel electrophoresis reveals the SSBs introduced by the DNA
1352 repair enzymes. The addition of dNTPs to the extracts would allow to study DNA
1353 synthesis/ligation capacity in parallel to DNA incision activity.
1354 Figure 3: Correlation between repair activities in extracts from human lymphocytes taken at
1355 different dates (approximately 2 months apart) and analysed using the BER (A) and NER (B*) in*
1356 *vitro* repair assay. Taken from [32], with permission.
1357
1358 Table 1. Overview of human DNA repair mechanims. Taken from [3], with permission.
1359
1360 Table 2. Correlation coefficients, R, for repair rates of 33 individuals at different sampling times
1361 (blood samples were taken approximately 4 weeks apart). * $p<0.05$. Adapted from [32], with
1362 permission.
1363
1364 Table 3. Inter-individual variation in repair activity using different assays. Data from samples
1365 taking at two occasions were available; mean values were used to estimate the range. Adapted
1366 from [12], with permission.
1367
1368 Table 4. Studies on association between age and DNA repair activity in leukocytes,
1369 lymphocytes or peripheral mononuclear blood cells.
1370
1371 Table 5. Studies on association between dietary factors and DNA repair activity in leukocytes,
1372 lymphocytes or peripheral mononuclear blood cells.
1373
1374 Table 6. Sources of exfoliated cells that can be collected in human biomonitoring studies
1375
1376

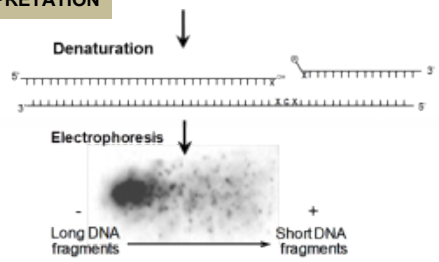

or
Cell culture
Blood cells
In vitro exposure
T0
T1
T2
T3
T4
Harvesting cells over time
Cells embedded in gel
Immersion of the cells in lysis solution
Cell lysis
washing
Nucleoids in gel
Optional: Incubation with lesion-specific enzymes
e.g. FPG, Endo III, T4EndoV
Incubation at 37°C
-
+
Denaturation / electrophoresis
NEUTRALIZATION
WASHING
STAINING
Automated comet analysis
Buffer = background control
Enzyme = high damage
Enzyme = low damage
PRINCIPLE & DATA INTERPRETATION
DNA damage
T0
T1
T2
T3
T4
Time



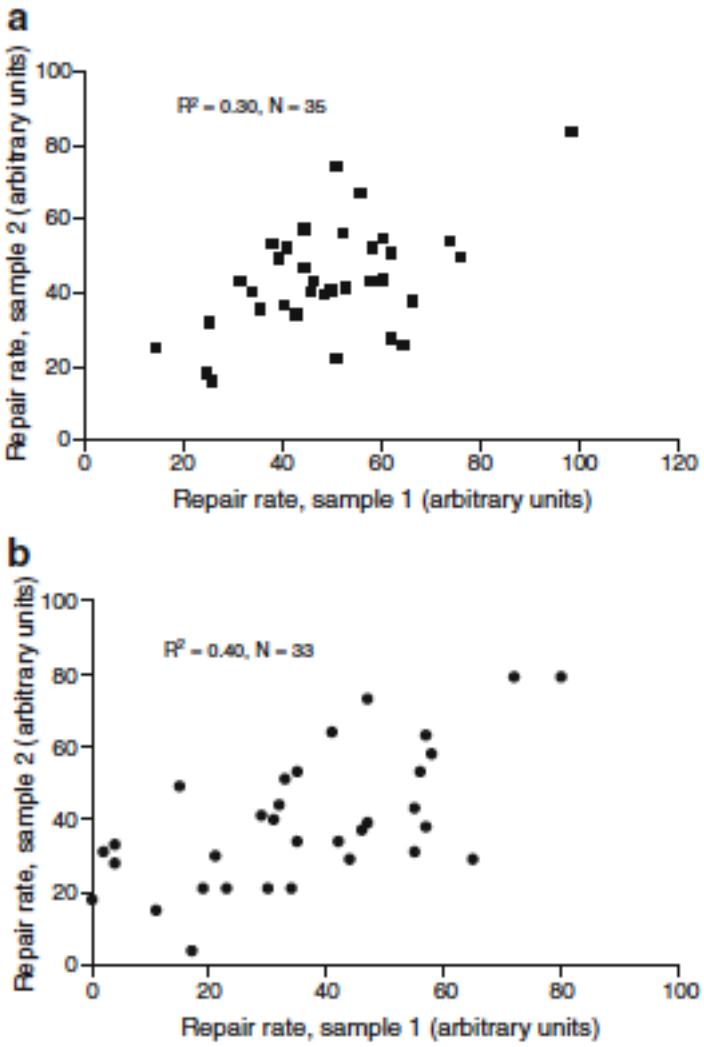
PRINCIPLE & DATA INTERPRETATION



DNA damage recognition and incision during incubation step at 37°C



↑ DNA in tail = ↑ Incision activity


a
Repair rate, sample 2 (arbitrary units)
R^2 = 0.30, N = 35
0
20
40
60
80
100
120
Repair rate, sample 1 (arbitrary units)
b
Repair rate, sample 2 (arbitrary units)
R^2 = 0.40, N = 33
0
20
40
60
80
100
Repair rate, sample 1 (arbitrary units)

Repair pathway	Damage repaired	Sources of damage
Direct reversal	Alkylated base O^6 -methyl-guanine, pyrimidine dimers (by photolyase)	Alkylating agents, nitrosoureas, streptozotocin, UV(C) light
Base excision repair	Oxidised bases, alkylated bases, abasic/apurinic/apyrimidinic sites, single-strand breaks	Reactive oxygen species (ROS), alkylating agents, ionizing radiation, spontaneous hydrolysis
Nucleotide excision repair	Bulky helix-distorting lesions, intra-strand cross links, DNA-protein cross links, inter-strand cross links	UV(C) light, cigarette smoke, dietary factors (aflatoxin, poly-aromatic hydrocarbons (benzo(a)pyrene))
Mismatch repair	Mismatched base pairs, small insertion loops	Replication errors, minor base modifications (oxidation, alkylation)
Double-strand break repair (i.e., homologous recombination and non-homologous end-joining)	Double-strand breaks	Ionising radiation, replication errors

	T=0	T ≈ 4 weeks	T ≈ 8 weeks	T ≈ 12 weeks	T ≈ 16 weeks
BER					
T ≈ 4 weeks	0.25				
T ≈ 8 weeks	0.42*	0.11			
T ≈ 12 weeks	0.40*	0.50*	0.30		
T ≈ 16 weeks	0.60*	0.08	0.62*	0.38*	
T ≈ 20 weeks	0.50*	0.35*	0.12	0.32	0.47*
NER					
T ≈ 4 weeks	0.40*				
T ≈ 8 weeks	0.32	0.60*			
T ≈ 12 weeks	0.51*	0.64*	0.52*		
T ≈ 16 weeks	0.44*	0.54*	0.48*	0.40*	
T ≈ 20 weeks	0.45*	0.42*	0.18	0.31	0.59*

Lesion repaired	Assay	N	Range (fold)	Source of variation	Reference
AP-sites	Plasmid	10	2.5	*Healthy individuals (age: 25-48 years). Authors did not correlate repair with other parameters.*	[18]
8-OxoG	Oligonucleotide	34	2	*Healthy individual (age: 18-60). There was no difference in OGG1 activity due to gender and smoking behaviour. Authors did not report age effect. OGG1 polymorphism not associated with altered OGG1 activity.*	[70]
8-OxoG	Oligonucleotide	120	2.8	*Healthy individuals. No significant differences between males and females, or between smokers and non-smokers. OGG1 activity was significantly lower in males older than 55 years compared to younger subjects. This effect was not observed in females.*	[24]
8-OxoG	In vitro comet assay	35	3	*Healthy individuals from intervention study (no effect of the intervention on DNA repair capacity). No further data on individuals' characteristics reported. Authors did not correlate repair with other parameters.*	[32]
8-OxoG	In vitro comet assay	40	41	*Individuals from 18 to 30 years old. Association between endogenous SBs and BER was not observed. Authors did not correlate repair with other parameters..*	[71]
UV-induced damage	Host cell reactivation assay (HCRA): catalase and luciferase assay	102	4.7 (luciferase assay) 7 (catalase assay)	*Healthy subjects (age: 19-79). Authors did analyze correlation with age or other factors.*	[72]
UV-induced damage	In vitro comet assay	33	7	*Healthy individuals from intervention study (no effect of the intervention on DNA repair capacity). No further data on individuals' characteristics reported. Authors did not correlate repair with other parameters.*	[32]
UV-induced damage	Host cell reactivation	63	11	*Individuals from an intervention study (age: 18-30, no effect of the intervention on DNA repair capacity). NER capacity was inversely associated with age, endogenous DNA SBs and BMI (adiposity).*	[73]
Benzo(a)pyrene	In vitro comet assay	8	10	*Healthy individuals; no further data. Authors did not correlate repair with other parameters.*	[31]
BPDE- induced damage	Aphidicoline-block cellular comet assay	122	40	*Healthy people (age: 19-48, cropreserved lymphocytes). Authors did not correlate repair with other parameters.*	[51]

Country (age) number of females (F) and males (M)	Cell type	Comet repair assay (substrate)	Effect on DNA repair biomarker	Adjustment	Reference
Slovakia (21-88 years, 227 M, 161 F) recruited from factories with occupational exposure to asbestos, stone wool or glass fibres and controls matched for age, sex, alcohol consumption and smoking	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation)	Positive association between age and repair incision activity (r = 0.1, P<0.05, correlation analysis not specified)	No control for confounding (with regard to age-dependent effects on DNA repair incision activity)	[69]
UK (young (20-35 years), middle (63-70 years) or old (75-82 years), n = 97, MF). Sampled from areas near Bristol, London, Wisbech, Aberdeen and Dundee	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells 10 min incubation)	Positive association between age and repair incision activity (r = 0.25, P = 0.06, Pearson correlation). Statistically significant group difference when tested by ANOVA	No control for confounding	[82]
Czech Republic (41 ± 11 years, 183 M, 61 F) recruited in local administration, medical centres and various branches of plastic industry	Lymphocytes (fresh for repair of DNA strand breaks, frozen for *in vitro* repair assay)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation) Repair of gamma radiation induced DNA strand breaks (5 Gy)	No association between age and repair incision activity and gamma radiation-induced DNA strand breaks (results not shown, type of control for confounding not specified in detail)	Analysed by both simple and multivariate regression analysis	[83]
USA (30-64 years, 48F, 48M) of White and African America race	PBMCs (frozen)	Repair of gamma radiation induced DNA strand breaks (6.3 Gy)	Positive association between age and repair activity in White females (r = 0.55, P < 0.01) and borderline statistical significance (r = -0.40, P = 0.06, linear regression) in African-American females. No effect in White or African-American males	Matched in sex and race strata	[84]

Denmark (18-83 years, 40 M, 38 F) from a national health survey in Copenhagen	PBMCs (frozen)	*In vitro* repair ($KBrO_3$, THP-1 cells 45 min incubation)	Inverse association between age and repair incision activity in women, but not in men. Decline in repair activity per year was 0.65% per year (95% CI: 0.16% – 1.14%) in multivariate regression analysis	Sex, body mass index (or waist-hip ratio), blood pressure, cholesterol, triglycerides, Hb1Ac, C-reactive protein, smoking and alcohol	[30]

Country (age) number of females (F) and males (M)	Cell type	Comet repair assay (substrate)	Effect on DNA repair biomarker	Effect on phytochemical	Reference
Sequential study of non-smokers (20-50 years, n = 6, M, Scotland) ingesting 100 mg/day of CoQ_{10} for 1 week and a subsequent washout period of 1 week	Lymphocytes (fresh)	*In vitro* repair (Ro19-8022 + light, lymphocytes, 20 min incubation)	Increased repair incision activity after supplementation compared to pre-supplementation. Decreased levels compared to supplementation period, although not statistically significant, after 1 week washout period	Increased CoQ_{10} concentration in plasma	[92]
Cross-over study on healthy non-smoking subjects (26-54 years, n = 14, MF, Scotland) ingesting 1, 2 or 3 kiwifruits/day for 3 weeks	Lymphocytes (fresh)	*In vitro* repair (Ro19-8022 + light, Hela cells, 10 min incubation)	Increased repair incision activity after consumption of kiwifruits (similar effect of 1-3 kiwifruits/day)	Increased plasma concentration of vitamin C concentration	[93]
Placebo-controlled parallel trial on non-smokers (18-50 years, n = 20, UK) ingesting tablets with α-carotene (3.7 mg) and β-carotene (8.2 mg) for 3 weeks	Lymphocytes (fresh)	Repair of H_2O_2 induced DNA strand breaks (100 µM)	DNA repair of DNA strand breaks over a 4 h incubation period (no repair in cells from the placebo group). Groups of subjects with intake of cooked carrots, mandarin oranges and vitamin C tablets were included in the study, but the results are not reported (risk of reporting bias)	Increased plasma concentration of β-carotene	[94]
Placebo-controlled parallel trial on non-smokers (20-60 years, n = 61, MF, UK) ingesting folic acid (1.6 mg/day) for 12 weeks	Lymphocytes (not specified)	*In vitro* repair (Ro19-8022 + light, CHO cells 20 min incubation)	Unaltered levels of repair incision activity in the whole study population. A restricted analysis of the quartile with lowest baseline red cell folate concentration showed a reduction of repair incision activity in the folate	Increased 5-methyltetrahydrofolate concentration in plasma, erythrocytes, and lymphocytes	[90]

			supplementation group (risk of bias due to subgroup analysis and unequal baseline folate concentration between supplementation and placebo group)		
Cross-sectional study of (young (20-35 years), middle (63-70 years) or old (75-82 years), n = 97, MF) from areas near Bristol, London, Wisbech, Aberdeen and Dundee	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells 10 min incubation)	Inverse correlation between plasma concentration of lutein/zeaxanthin (r = -0.31, P = 0.06, Pearson correlation). Marginally positive association with retinol (r = 0.25, P = 0.06). No correlation with vitamin C, β-carotene, lycopene and α-tocopherol. No control for confounding	Not applicable	[82]
Placebo-controlled parallel trial on smokers (39 ± 12 years, n = 48, M, Denmark) ingesting 500 mg vitamin C and 182 mg vitamin E per day for 4 weeks	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Increased repair incision activity in the group of subjects who ingested vitamin C and E as slow-release tablets. No effect in the group that received tablets with fast-release tablets	Increased vitamin C in plasma after ingestion of both slow- and fast-release tablets	[39]
Placebo-controlled parallel trial on non-smokers (27 ± 6 years, n = 43, MF, Denmark) ingesting 600 g fruit/vegetables or tablets with the corresponding amount of vitamins and minerals for 4 weeks	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Unaltered levels of repair incision activity	Strong decrease in plasma vitamin C in the placebo group. Increased lycopene levels (fruit/vegetable group) and β-carotene (tablet group)	[39]
Sequential study of non-smokers (18-45 years, n =	Lymphocytes (frozen)	*In vitro* repair (benzo[a]pyrene-	Unaltered levels of repair incision activity	Not reported	[60]

36, MF, Netherlands), selected according to ERCC1 genotype, ingesting blueberry and apple juice for 4 weeks after a 5-day washout period		diolepoxide, cells or incubation not reported)			
Placebo-controlled parallel trial on non-smokers (18-30 years, n = 48, MF, UK) ingesting a supplement (100 µg Selenium, 450 µg vitamin A, 450 µg retinol, 90 mg vitamin C and 30 mg/ vitamin E) for 6 weeks	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, Hela cells, 20 min incubation)	Unaltered levels of repair incision activity	Not reported	[71]
Cross-over study on smokers (22 ± 3 years, sex not specified, Italy) ingesting steamed broccoli (250 g/day) for 10 days	PBMCs (frozen)	*In vitro* repair (Ro19-8022 + light, A549 cells, 20 min incubation)	Unaltered levels of repair incision activity	Increased plasma concentration of folate and lutein. Unaltered levels of β-carotene	[91]
Placebo-controlled parallel trial on smokers (45-75 years, n = 69, M, Norway) ingesting kiwifruit or a phytochemical-rich diet for 8 weeks	Lymphocytes (frozen)	*In vitro* repair (Ro19-8022 + light, substrate cell not reported, 20 min incubation; UV-C, substrate cell not reported, 30 min)	Increased base excision (Ro19-8022 + light) and decreased nucleotide excision (UV-C) repair in both kiwifruit and phytochemical-rich diet group	Increased vitamin C (both groups). Increased β-carotene and tocopherol in the phytochemical-rich group	[62]

Target tissue	Surrogate tissue	How to obtain cells
Bladder	Exfoliated epithelial cells	• Isolate from urine
Upper respiratory tract	Buccal cells Nasal epithelial cells Mouth cells	• Mouth rinse or scraping • Nasal lavage • Isolate from saliva
Lower respiratory tract	Lung derived cells	• Isolate cells from induced or spontaneously produced sputum • Broncho-alveolar lavage
Colon	Exfoliated epithelial cells	• Isolate from stool
Mammary	Exfoliated epithelial cells	Isolate from • Nipple aspirate • Ductal lavage • Breast milk
Prostate / testis	Epithelial cells spermatozoa	• Isolate from ejaculate
Other tissues		• Biopsy (invasive)